

Worcester Polytechnic Institute Digital WPI

Major Qualifying Projects (All Years)

Major Qualifying Projects

April 2016

The SERCA3 Pathway is Correlated to Optimal Insulin Secretion in Pancreatic Cell Lines Derived From Type-2 Diabetic Patients

Megan Robidas

Worcester Polytechnic Institute

Victor Chau

Worcester Polytechnic Institute

Follow this and additional works at: <https://digitalcommons.wpi.edu/mqp-all>

Repository Citation

Robidas, M., & Chau, V. (2016). *The SERCA3 Pathway is Correlated to Optimal Insulin Secretion in Pancreatic Cell Lines Derived From Type-2 Diabetic Patients*. Retrieved from <https://digitalcommons.wpi.edu/mqp-all/1759>

This Unrestricted is brought to you for free and open access by the Major Qualifying Projects at Digital WPI. It has been accepted for inclusion in Major Qualifying Projects (All Years) by an authorized administrator of Digital WPI. For more information, please contact digitalwpi@wpi.edu.

The SERCA3 Pathway is Correlated to Optimal Insulin Secretion in Pancreatic Cell Lines Derived From Type-2 Diabetic Patients

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degrees of Bachelor of Science

In

Biochemistry (VC) and Biology/Biotechnology (MR)

By

Victor Chau

Megan Robidas

April 28, 2016

APPROVED:

Agata Jurczyk, PhD
Program in Molecular Medicine
UMass Medical School
MAJOR ADVISOR

David Adams, PhD
Biology and Biotechnology
WPI Project Advisor

This report represents the work of WPI undergraduate students submitted to the faculty as evidence of completion of a degree requirement. WPI routinely publishes these reports on its website without editorial or peer review. For more information about the projects program at WPI, please see <http://www.wpi.edu/academics/ugradstudies/project-learning.html>

ABSTRACT

The high prevalence of Type 2 diabetes (T2D) has brought it to the foreground of attention in the scientific community. SERCA3-ATPases help regulate intracellular Ca^{2+} levels, which are critical for maintaining normal insulin secretion and thus glucose homeostasis. SERCA3 down-regulation has been observed in T2D patients, but it is currently unclear whether this down-regulation is a cause or consequence of T2D. To investigate the role of the SERCA3 pathway in diabetes, experiments in this project knocked down the SERCA3 gene in EndoC- β H1 human pancreatic islets derived from diabetic patients and from pancreatic beta cells derived and differentiated from human stem cells. This was done in order to determine if the knockdowns would affect insulin secretion or induce physiological changes in pancreatic beta cells. Western blot analysis and fluorescence intensities were used to determine relative protein expression. qPCR experiments were performed to determine mRNA levels. In addition, the fluorescence intensities of SERCA3 and insulin in healthy and T2D pancreatic islets were quantitated. Protein levels of insulin and SERCA3 were significantly lower in diabetic pancreatic islet samples compared to healthy controls. Two-tailed t-tests for SERCA3 and insulin down-regulation gave p values of 2.01×10^{-8} and 6.26×10^{-3} , respectively. Western analysis of the SERCA3 and insulin levels in healthy and T2D individuals also confirmed downregulation of both proteins in T2D islets compared to healthy islets as observed by immunofluorescence. Adenovirus-shRNA-induced knockdown of SERCA3 expression was validated at both the mRNA (q-RT-PCR) and protein (Western blots) levels, but those cells have not yet been assayed for the potential effects of insulin secretion. Overall, the

results of this project indicate that the SERCA3 pathway is important for optimal insulin secretion.

TABLE OF CONTENTS

Title Page	i
Abstract	ii
Table of Contents	iv
Acknowledgements	v
Background	1
Overview on Diabetes	1
Insulin and Glucagon Signal Transduction Pathways	1
Alteration of Cell Signaling in Type-2 Diabetes	5
TCP-1 and CCT	8
SERCA3 and Type-2 Diabetes	11
Project Purpose	15
Methods	16
Selection of Human Pancreatic Tissue nPOD Samples	16
Human β -Cell Growth, Fixation, and Staining	17
qRT-PCR	19
Viral Knockdowns and Cell Culturing	19
Immunofluorescent and Immunohistochemistry Staining	20
Western Blotting	21
Results	23
Human β -Cell Immunohistochemical Staining Shows Decreased of SERCA3 in T2D Patients	24
Insulin and SERCA3 Immunofluorescence	25
SERCA3 Western Blots	29
Adenoviral Knockdown Experiments for SERCA3	31
SERCA3 Knockdown	33
Discussion	35
Bibliography	38

ACKNOWLEDGEMENTS

We would first like to thank Dale Greiner, Ph.D. and Agata Jurczyk, Ph.D., in the Program of Molecular Medicine at the University of Massachusetts Medical School for allowing us the opportunity to work in their lab. Agata has been extremely accommodating and helpful throughout this project, and has provided continuous support in teaching us many different fundamental skills that have helped us become better researchers. We would also like to thank the Department of Pathology at the University of Florida for graciously providing the human pancreatic sections used in this project, which are from the Network for Pancreatic Organ Donors with Diabetes (nPOD). The stem cell-derived β -cells were generously provided by Melton Lab (Harvard University). We would also like to thank Professor Destin Heilman Ph.D., the UMass MQP Project Center Advisor, for maintaining the shuttle services to and from WPI to UMMS throughout the year. Lastly, we really want to thank Professor David Adams Ph.D., our WPI Project Advisor, for his immense help in setting up the project, amazing feedback and guidance throughout the year, and help in editing the final MQP report.

BACKGROUND

Overview of Diabetes

Diabetes is a glucose dysregulation disease that has become a serious global health problem affecting approximately 8% of the adult population worldwide, and is currently at the foreground of attention in the scientific community (Gilon et al., 2014). The development of type 2 diabetes (T2D) is of special heightened concern, as it occurs approximately 20 times more frequently than type 1 diabetes (T1D) (Gilon et al., 2014). While T1D causes self-destruction of insulin-producing cells in the body, T2D develops when beta cells fail to secrete enough insulin to compensate for relative insulin deficiency. T2D is the more prevalent type, with 285 million cases recorded worldwide as of 2010 (Williams, 2011). T2D has a genetic risk, but dietary habits and environmental factors also influence this disorder. Only about 1-2% of all T2D cases are caused by a single gene mutation (Gilon et al., 2014). T2D patients usually have insulin resistance, a lack of response to insulin, or lack insulin altogether, and tend to develop hyperglycemia. Although much research has focused on mechanisms of insulin secretion, factors leading to insulin insensitivity are not fully understood. For instance, the exact mechanism of how beta cells fail to secrete enough insulin is unknown, or why peripheral tissues become insulin insensitive.

Insulin and Glucagon Signal Transduction Pathways

The human endocrine system consists of various glands that secrete hormones into the circulatory system to interact with target organs (Williams, 2011). The system functions to help maintain homeostasis in the body, and includes a variety of hormones such as testosterone, estrogen, insulin, glucagon, and epinephrine. Hormones help regulate

every essential function in the body, and are paired with alternative hormones to check and balance the function of the first. The hormones often bind receptors present on the target tissues to initiate signaling pathways that alter cell function.

With respect to diabetes, the main hormones of interest that help regulate glucose homeostasis are insulin, glucagon, and somatostatin. Insulin is the body's only hormone for lowering serum glucose levels. Following the ingestion of a meal, glucose levels increase in the blood. Insulin is secreted from the pancreatic β -cells into the bloodstream and binds to insulin receptors present on target tissues. The binding of insulin to its target receptor (**Figure-1**, blue receptor, right side) initiates a signaling pathway(s) to upregulate glucose transporters (GLUT) (red receptor, left side) to the cell surface. The GLUT's bind serum glucose and transport it inside the cell to participate in glucose metabolism.

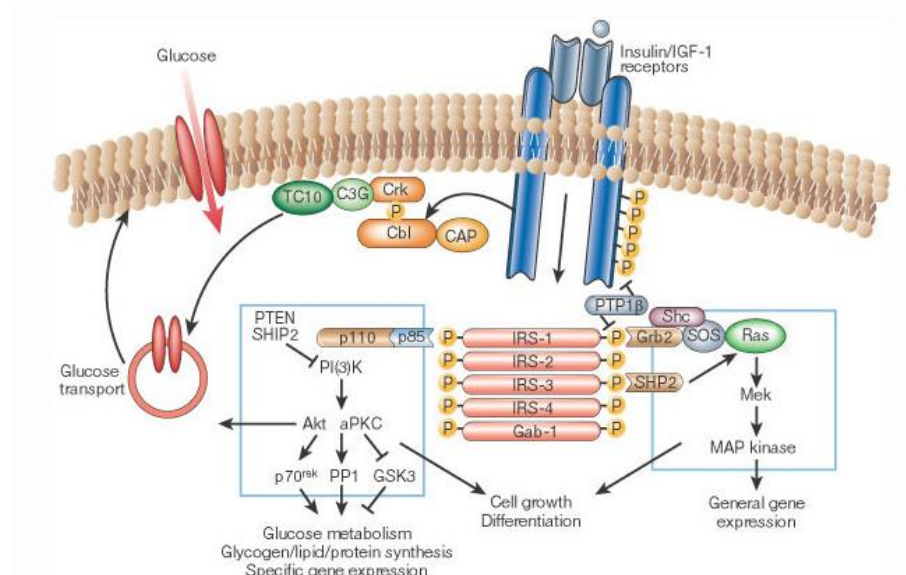


Figure-1: Diagram of Insulin Receptor Signal Transduction Pathways. Shown are the major pathways known to be activated by the binding of insulin to its receptor (blue receptor, right side), including the Ras/MAP kinase pathway (right side blue box), PI(3)K/AKT pathway (left side, blue box), and Cbl/CAP/TC10 pathway (orange and green proteins shown near the membrane). Diagram is from Saltiel and Kahn (2001).

The up-regulation of GLUT in response to insulin is one of the best characterized cell signaling pathways. The binding of insulin to its receptor induces the phosphorylation of cellular proteins, such as members of the insulin receptor substrate (IRS) family (red, diagram center), Shc (purple, diagram right), and Cbl (orange, diagram center). These phosphorylated proteins then activate a diverse series of signaling pathways, including the PI(3)K and Akt kinases (left side, blue box), Ras and the MAP kinase cascade (right side, blue box), and the Cbl/CAP/TC10 pathway (green and orange proteins shown immediately beneath the cell membrane). These pathways act together to regulate glucose, lipid, and protein metabolism.

The regulation of insulin secretion from the pancreas is a complex process affected by a variety of stimuli including nutrients, hormones, neurotransmitters, and drugs (Rorsman and Braun, 2013). Insulin is produced and released from pancreatic β -cells. The main trigger for insulin secretion is elevated serum glucose, which induces a biphasic pattern of insulin release. The first phase involves a rapid release of insulin within minutes of exposure to elevated glucose, followed by a more extended second phase of insulin release. The first phase of insulin secretion appears to be lost in some T2D patients, leading to diminished insulin secretion and hyperglycemia. Although scientists are still researching this complex homeostatic process, the main steps (**Figure-2**) are believed to include several processes: 1) the transport of glucose inside pancreatic β -cells using receptor GLUT-2, 2) cellular metabolism of the glucose to ATP using glycolysis and the TCA cycle, 3) the elevation of the ratio of ATP to ADP which induces closure of cell-surface ATP-sensitive K^+ (KATP) channels, leading to cell membrane depolarization, 4) the opening of cell surface voltage-dependent Ca^{2+} channels (VDCC) allowing Ca^{2+} to

increase in the cell (Gilon et al., 2014), and 5) increased insulin exocytosis triggered by elevated cytoplasmic Ca^{2+} levels.

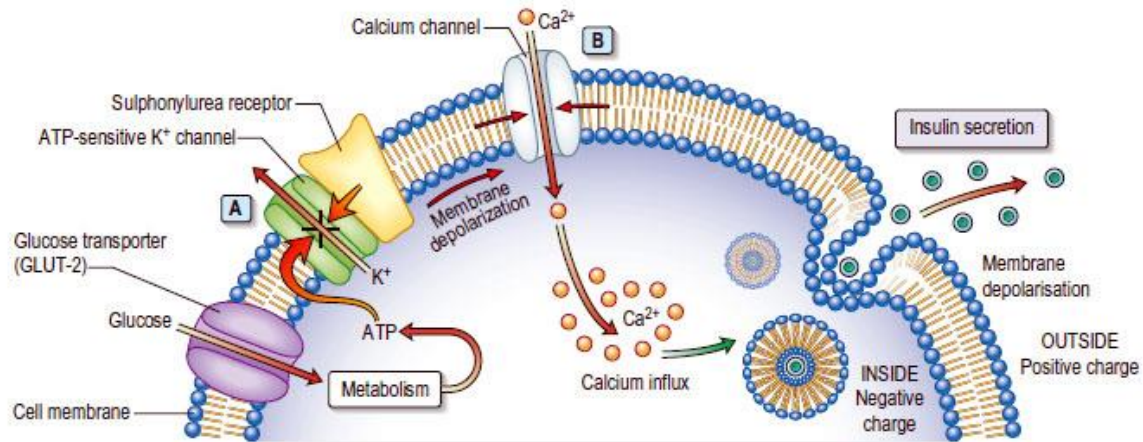


Figure-2: Diagram of the Main Steps Controlling Insulin Secretion from a Pancreatic Beta Cell. Shown in the diagram is the entrance of glucose into the cell using a glucose transporter (purple receptor), the metabolic production of ATP from the glucose, closure of the ATP-sensitive K⁺ channel (green receptor), membrane depolarization, the flow of Ca²⁺ into the cell via a calcium channel, activation of Ca²⁺ dependent phospholipid protein kinase, and exocytosis of insulin granules (diagram right side). (<http://www.medicinehack.com/2011/08/insulin-secretion-local-regulation.html>)

Glucagon is a hormone involved in glucose homeostasis. Glucagon is produced in pancreatic alpha islet cells and results in the storage of glucose in the liver as glycogen. Once stored, glycogen can be converted back into glucose to stimulate insulin activity. The reintroduction of glucose can also raise blood sugar levels during periods of starvation. Somatostatin, produced in pancreatic delta islet cells, balances the function of glucagon by inhibiting the release of glucagon from pancreatic alpha cells (Gilon et al., 2014). Glucagon, somatostatin, and insulin work in conjunction to maintain constant blood sugar levels throughout the body. Dysregulation of any of these hormones results in dysregulation of glucose homeostasis.

Alteration of Cell Signaling in Type-2 Diabetes

Disruption of any of the homeostatic pathways described above can result in glucose dysregulation and diabetes. Scientists are still investigating which pathways result in T2D, but several key processes are thought to be involved: 1) dysregulation of cell growth pathways leading to a lower number of pancreatic alpha/beta cells, resulting in lower insulin secretion, 2) disruption of the insulin signaling pathways, leading to insulin resistance and diminished GLUT up-regulation, and 3) disruption of the Ca^{2+} signaling in pancreatic cells, leading to reduced insulin secretion.

Cell proliferation pathways are important for cell differentiation and, if dysregulated, may result in diseases, such as T2D. For example, Neurogenin (Ngn) is a transcription factor which acts upstream in the NeuroD pathway, involved in pancreas development and nervous system differentiation. Current studies suggest that the Ngn3 transcription factor is the precursor to endocrine cells, which aids in cell elongation and cell growth (Bai et al., 2012). Inhibition of the Ngn-NeuroD cascade has been shown to prevent premature differentiation of endocrine cells (Jensen et al., 2000). More specifically, this 2000 study looked into Notch1 and its ability to inhibit this cascade (Jensen, et al., 2000). This is an example of dysregulation of the Ngn-NeuroD pathway, which has been confirmed to inhibit differentiation and cause loss of function in pancreatic alpha/beta-cells.

With respect to the GLUT pathway discussed above, some scientists believe that a disruption of insulin signaling results from chronic long-term ER stress in cells that use glucose, resulting in a lack of sensitivity to insulin and a lack of GLUT placement on the cell surface (Ozcan et al., 2004; Nakatani et al., 2005). Chronic ER stress stems from the increased cellular demand for insulin, which results in increased insulin production.

Treatment with chaperone proteins that help alleviate ER stress has been tested in diabetic patients. The cell's unfolded protein response (UPR) aims to halt protein translation, degrade misfolded proteins, and activate signaling pathways that activate chaperone proteins. Chemical chaperones that lower ER stress were found to restore insulin sensitivity and glucose homeostasis in a mouse model of T2D (Ozawa et al., 2005; Ozcan et al., 2006). Other scientists have shown that obesity (that strongly correlates with T2D) is associated with ER inflammation. Inflammation activates the c-Jun N-terminal kinase (JNK) and inhibitory kappa-B kinase (IKK) signaling pathways, leading to insulin insensitivity, lack of GLUT up-regulation, and hyperglycemia (Wellen and Hotamisligil, 2005).

With respect to the SERCA3 pathway discussed above, some scientists believe that disruption of Ca^{2+} signaling in pancreatic cells leads to reduced insulin secretion and T2D (Borge et al., 2002; Liang et al., 2014; Gilon et al., 2014). This MQP project focuses on the SERCA3 pathway, which is an important signaling pathway in Ca^{2+} signaling. This pathway appears to be affected in T2D. However, it is unclear whether the down-regulation of SERCA3 is a cause or consequence of the disease, or whether an irregular level of SERCA3 in humans affects physiological health (Zarain-Herzberg et al., 2014).

A functional homeostatic system requires all hormones to work optimally and have the capability to adjust and accommodate changing levels of stress. Any decrease in the system activity can result in possible system failure. Such physical changes may include: lower levels of alpha/beta islet cells, lower levels of insulin, and unregulated levels of glucose. These changes initiate the development of T2D. Short-term insulin treatment has been shown to cause a rise in Ca^{2+} levels and to stimulate insulin secretion in pancreatic β -cells (Xu et al., 2000). However, in the long term, insulin treatment for diabetic patients

causes a sustained increase in calcium, enhanced glucose-stimulated insulin secretion, and no successful increase in insulin levels. Early in T2D patients, pancreatic β -cells secrete more insulin to compensate for insulin resistance, but this results in hyperinsulinemia (excess insulin relative to glucose). Eventually, β -cell functioning deteriorates and can no longer compensate for the insulin resistance. Unfortunately, research has not determined which event occurs first, or what the progressions of these consequences are *in vivo*.

There is extensive research being done in the Program of Molecular Medicine at the University of Massachusetts Medical School in order to understand the mechanisms and pathways involved in T2D. Our mentor, Dr. Agata Jurczyk, focuses her research on the mechanisms of insulin secretion in pancreatic β -cells. The lab at UMMS has published numerous articles relating to T2D and its signaling pathways. Findings from the lab have provided evidence that human beta cells can proliferate under high glucose conditions; therefore, it is not the inability of human beta cells to proliferate that may lead to T2D but rather some other restriction that results in reduction of beta cell mass (Diiorio et al., 2011). It is important to note that in numerous experiments models of increasing rodent β -cell proliferation were often much more successful than human β -cell proliferation (Jurczyk et al., 2014). These experiments provide evidence that β -cells proliferate differently across species, and that diseases develop differently across species as well. However, animal models are still useful to study the progression of the disease including the onset and early development that is often impossible to study in people because we cannot predict when an individual will develop T2D.

Perhaps some of the most relevant and significant contributions include the discovery of improved function and proliferation of adult human β -cells in diabetic mice treated with alogliptin (Jurczyk et al., 2013). The insulin level in treated cells was about 10

times greater than in the control, which is consistent with the increase in human β -cell proliferation with alogliptin treatment and increased insulin secretion. Another discovery made by this lab is the relationship between individuals with schizophrenia and their higher rates of contracting T2D than the general population (Jurczyk et al., 2015). A final relevant contribution of the lab is the discovery of the absence or disorganization of cilia in pancreatic islets can lead to dysregulated insulin secretion, which may contribute to diabetic symptoms (Diiorio et al., 2014; Harris, 2013).

TCP-1 and CCT

While the focus of this project analyzes the role of the SERCA3 pathway and its implications in the development of diabetes, there are many other pathways involved in proper cell functioning such as protein folding. TCP-1 is one such pathway; impaired function of the TCP-1 chaperone protein may be linked to impaired function of insulin secretion. This presents the possibility that down-regulation of the TCP-1 pathway, like the SERCA3 pathway, may contribute to development of T2D. One of the functions of chaperone proteins in the cell is to help correctly fold proteins inside the ER. The T-Complex Protein-1 (TCP-1) is encoded by gene *tcp-1* located on the long arm of chromosome-6 (Fonatsch et al., 1987; Willison et al., 1987). TCP-1 is a member of the chaperonin containing TCP1 complex (CCT), also known as the TCP1 ring complex (TRiC). This complex consists of two identical stacked rings, each containing eight different proteins (**Figure-3**).

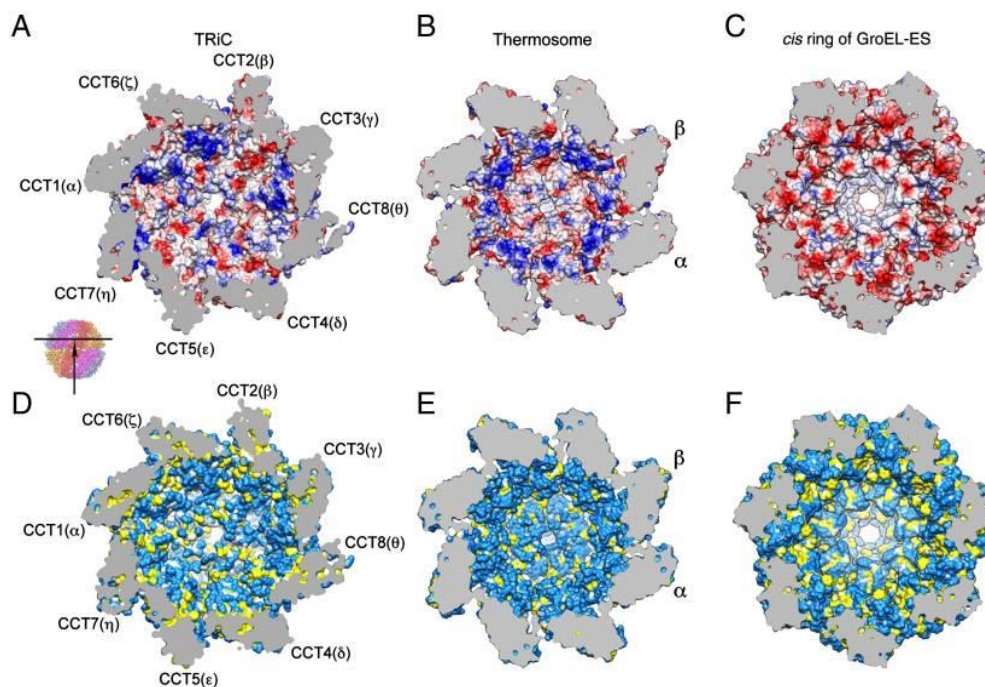


Figure-3: Diagram of the CCT Complex. Shown are the surface properties of protein TRiC (panel A) compared to similar proteins thermosome (panel B) and GroEL-GroES (panel C). Blue represents positively charged patches, red negatively charged patches, and white neutral patches. Panels D-F denote the inner wall surface properties of the same respective proteins. Blue denotes hydrophilic side chains, and yellow denotes hydrophobic side chains. Figure is from: Cong et al., 2010.

Unfolded polypeptides enter the CCT central cavity and are folded in a process dependent on ATP. CCT aids in protein folding and inhibits protein aggregation (Slavinotek et al., 2001). Many chaperone proteins are operated in an ATP-dependent manner which allows for the regulated binding and folding of native and synthesized proteins such as: actin, tubulin, and myosin (Slavinotek et al., 2001; NCBI TCP1 t-complex, 2016). Protein folding is critical for cell function because misfolded proteins cannot function optimally. Protein aggregation can also inhibit hormonal secretions by the cell.

An impaired loss of function of chaperone proteins may be responsible for diseases such as Bardet Biedl Syndrome (BBS). In this case, a lack of appropriate protein folding

results from incorrect assembly of the BBS-2 subunit of the “BBSome” chaperonin itself (Harris, 2013). BBSomes are chaperone proteins that interact with leptin receptors, a hormone receptor involved with body weight regulation (Seo, et al., 2010). Although no work has currently found a direct link to BBSomes and T2D, a prediction can be made that impaired function of TCP-1 chaperone protein might be linked to an impaired function of insulin secretion. Impaired chaperone function leads to BBS, and patients share many symptoms with those of T2D patients (Harris, 2013). Previous research has shown that the knockdown of the BBS2 subunit gene in mice significantly lowers chaperone protein function as well as the ability to recover from an elevated glucose challenge (Harris, 2013).

CCT belongs in the Group II category of chaperonin proteins. Unlike Group I chaperonins, the entire complex of Group II chaperonins usually contains two stacked, octa- or nonameric rings and six to nine different subunits (Slavinotek et al., 2001). This category of chaperone proteins are defined as regulator proteins that function without the use of a co-chaperone, such as GroES, which in prokaryotes works in conjunction with GroEL. Eukaryotes share a parallel to Group-I chaperone proteins with prokaryotes, existing as Hsp10 with Hsp60 within eukaryotic organisms (Slavinotek et al., 2001). Co-chaperones function to cap the protein at the apical domain in the complex during protein folding (Chen et al., 2011). This cap is important in Group I chaperone proteins in order to maintain a highly basic microenvironment for the protein to be forced into its native conformation. However, Group II chaperone proteins that do not operate with a closely related co-chaperone. In place of a co-chaperone, this group of chaperones instead uses a helical protrusion above the apical domain that essentially performs this same function (Chen et al., 2011).

It is important for scientists to understand the functions of various CCT subunits, because small molecular changes can lower CCT chaperone function and lower hormonal secretion. Previous research has been done on the CCT β subunit in mice (CCT complex, subunit 2 / subunit β) as well as CCT δ subunit in yeast (CCT complex, subunit δ / subunit 4), and have concluded that both of these subunits can affect cell contractility, proliferation, migration of F-actin, and also induce conformational shifts within the CCT δ apical domain, respectively (Chen et al., 2011; Spiess et al., 2015). Changes within the apical domain in subunit CCT δ in yeast has been shown to disrupt the allosteric function of CCT ATPase, disrupting the binding ability within both the equatorial and substrate-binding domains of the CCT complex altogether (Spiess et al., 2015).

SERCA3 and Type-2 Diabetes

Sarco(endo)plasmic reticulum Ca^{2+} transport ATPase-3 (SERCA3) is the main pathway examined in the experiments of this MQP project. SERCA's are a family of ATPases (SERCA-1, 2, 3) expressed in pancreatic islets. SERCA's are intracellular pumps located in the sarcoplasmic or endoplasmic reticulum of cells. In muscle cells, SERCA3 uses ATP to help move Ca^{2+} from the cytosol to the sarcoplasmic reticulum lumen, and sequesters Ca^{2+} during muscular contraction (Dode et al., 1996). In pancreatic cells, immunocytochemistry showed that SERCA3 is restricted to β -cells in the mouse pancreas (Arredouani et al., 2002). The same researchers used SERCA-knockout mice to study β -cell cytosolic free Ca^{2+} , insulin secretion, and glucose homeostasis. SERCA knockouts showed that SERCA2b, but not SERCA3, is involved in basal Ca^{2+} regulation in β -cells, and SERCA3 functions in Ca^{2+} oscillations in response to glucose. However, their data

showed that a lack of SERCA3 by itself is insufficient to alter glucose homeostasis or impair insulin secretion in mice (Arredouani et al., 2002).

Because SERCA3 alters cytoplasmic Ca^{2+} beyond background levels in response to glucose, loss of SERCA3 may contribute to T2D. The secretion of insulin from pancreatic β -cells is dependent upon Ca^{2+} levels (Varadi et al., 1999), and disrupted Ca^{2+} homeostasis has long been established as a hallmark of diabetes. Thus, mutations within the gene encoding SERCA3 might weaken the β -cell's response to glucose, and may contribute to diabetic symptoms. In contrast to the mouse KO study discussed above that showed no alteration in insulin secretion in KO mice (Arredouani et al., 2002), other scientists have shown that SERCA3 ablation decreases glucose-induced insulin release, which would logically contribute to diabetic symptoms (Ravier et al., 2011). Impaired SERCA3 function has previously been correlated with β -cell apoptosis in experiments using diabetic animal models (Varadi et al., 1999). And it has previously been shown that down-regulation of SERCA3 can affect calcium homeostasis in pancreatic β -cells, which impairs normal insulin secretion (Zarain-Herzberg et al., 2014). Most importantly, Varadi et al. (1999) identified four rare missense mutations in exons 4, 14, and 15 in the SERCA3 gene of T2D patients with marked hyperglycemia and β -cell dysfunction. They concluded that in white Caucasians, the SERCA3 locus might contribute to the genetic susceptibility to T2D.

However, it is currently unknown if SERCA3 down-regulation is a cause or an effect of the development of T2D. The lab at UMMS identified a significant downregulation ($p = 0.0071$) of SERCA3 in beta cells from T2D patients compared to healthy patients. Thus, the SERCA3 encoding exons become excellent candidates to attempt to correlate mutations with pancreatic β -cell defects. For these reasons, our

experiments were designed to test the effects of knocking down SERCA3. Specifically, the knockdowns were performed for the ATP2A3 gene. Genes ATP2A 1-3 encodes 11 different isoforms of the SERCA pumps. More specifically, gene ATP2A3 encodes isoforms SERCA3 a-f through alternative splicing (Zarain-Herzberg et al., 2014). SERCA3 isoforms are present in hematopoietic cell lines, which give rise to all other blood cells, while SERCA2b and SERCA3 are the main isoforms present in pancreatic β -cells. Previous experiments have shown that impaired glucose-stimulated insulin secretion is associated with reduced expression of isoforms SERCA2b and SERCA3 (Zarain-Herzberg et al., 2014). Thus, down-regulation of these pathways may affect Ca^{2+} homeostasis in pancreatic β -cells and impair insulin secretion. Previous studies have also shown that improved glucose homeostasis is correlated with up-regulation of several islet genes including ATP2A3 (encoding SERCA3 isoforms a-f), indicating that mutations in those proteins could cause a lowering of insulin secretion due to changes in Ca^{2+} regulation (Zarain-Herzberg et al., 2014). This is important because alteration in Ca^{2+} regulation is often seen in heart failure, one of the leading causes of death in diabetic patients. Much remains unknown regarding early changes in Ca^{2+} regulation in a diabetic heart, but it has been shown that exercise improves sarcoplasmic reticulum Ca^{2+} regulation through the SERCA pathway, thus showing that lifestyle choices can have an impact on diabetes (Zarain-Herzberg et al., 2014).

Another very similar pathway to SERCA3 is the Ca^{2+} transport ATPase isoform SERCA2b. Down-regulation of SERCA2b has also been shown to affect calcium homeostasis in pancreatic β -cells, which would in turn impair normal insulin secretion. Notably, SERCA2b has a reputation of being more sensitive to calcium than its cousin SERCA3. However, specific mutations in exons 4, 14, and 15 of SERCA3 have been

shown to be present in T2D patients (Varadi et al., 1999) and mutations in exon-4 have been shown to affect transitions during the calcium transport cycle (Zarain-Herzberg et al., 2014), so it is possible that SERCA3 is more important in human than in rodent models and our project will focus on SERCA3. Prior to this project, as mentioned above, proteome studies of healthy and T2D patients' beta cells detected significant downregulation of SERCA3 in T2D patients (Jurczyk et al., unpublished data). In any case, an increased understanding of the various SERCA isoforms is generally important.

PROJECT PURPOSE

As discussed in the Background section, scientists believe that disruption of chaperone function or intracellular Ca^{2+} levels in pancreatic cells may lead to reduced insulin secretion and type-2 diabetes (T2D). This MQP project focuses on one protein that may be important to insulin secretion, regulation, and hormonal resistance: SERCA3. SERCA3 is localized in pancreatic β -cells and is known to help regulate intracellular levels of Ca^{2+} . This protein appears to be diminished in T2D patients, but it is unclear whether its down-regulation is a cause or consequence of the disease. Our project aims to confirm a decreased expression of SERCA3 in human T2D pancreatic samples relative to healthy controls, and to create a knockdown of the SERCA3 gene in human pancreatic cells to help determine whether its lack of functions may cause any intracellular physiological change.

The knockdowns will be accomplished by Adenovirus using RNAi technology in EndoC β H-1 human pancreatic cell lines and stem cell derived human pancreatic beta cells, and verified by techniques such as western blotting, immunofluorescence imaging, and qRT-PCR. We will also determine the role of SERCA3 in pancreatic beta cells. Finally, we will determine levels of insulin and SERCA3 expression in order to compare the differences of SERCA3 in healthy and T2D patients.

METHODS

Selection of Human Pancreatic Tissue nPOD Samples

The human pancreatic tissues used in this project were graciously provided to our group by the Department of Pathology at the University of Florida, and are a part of the Network for Pancreatic Organ Donors with Diabetes (nPOD) (www.jdfnnpod.org). Samples were chosen based on several factors, including the patient's age, BMI, and phenotype (Healthy or T2D). The healthy samples chosen for the control group were specified to be within a similar age group of 20-24 years and BMI range of 20.7-22.6, but with varying race and gender. There is more variance in age and BMI values in the experimental group due to the greater range in differences that exist among diabetic patients. The T2D samples chosen for the experimental group ranged from 45-57 years, with a BMI range of 28.1-30.4.

Table 1: Characterization of Healthy Control Pancreatic Tissues Based on Age, Sex, Race, and BMI.

	Controls: Healthy			
	Age	Sex	Race	BMI
6001	22	M	Caucasian	21.9
6029	24	F	Hispanic	22.6
6179	22	F	Caucasian	20.7
6235	23	M	African American	22.3
6238	20	M	African American	21.7

Table 2: Characterization of Diabetic Experimental Pancreatic Tissues Based on Age, Sex, Race, and BMI.

	Experimentals: Type-2 Diabetic			
	Age	Sex	Race	BMI
6127	45	F	Caucasian	30.4
6147	47	M	Hispanic	29.8
6199	53	M	African American	29.6
6255	55	M	Caucasian	29.4
6272	57	F	African American	29.6
6277	48	M	African American	29.5
6280	47	M	African American	28.1
6283	56	F	Caucasian	28.1

13 nPOD samples were used throughout the experiments of this project, 5 of which were from healthy individuals, and 8 of which were from Type-2 diabetic patients. This ensured that the total sample sizes used to collect experimental results were big enough to conduct viable T-tests.

Human β -Cell Growth, Fixation, and Staining

EndoC- β H1 Human Beta Cells were supplied by Endocells, and were grown in DMEM Low Glucose media (Life Technology, Cat.#11885-084) supplemented with Sodium Selenite (6.7 μ g/mL), Transferrin (5.5 mg/mL), Nicotinamide (1.2 g/mL), Albumin (10 g), and BME (1.75 μ L into 500 mL media) according to the Endocells protocol (Paris, France). The coverslips and culturing dishes were coated with coating media DMEM High Glucose (Life Technology, Cat. #11965-092) and supplemented with

ECM (Sigma-Aldrich®, Cat. #E1270), Pen-Strep, and Fibronectin from bovine plasma (Sigma-Aldrich®, Cat. #E1270). The cells were split and passaged every 2 weeks and the media was changed once a week.

For immunofluorescence, the cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Fixed coverslips were first washed with 1x PBS Solution (Life Technologies, Cat.#14190-144) and blocked in custom-made PBS-AT Solution using 500 mL PBS, 10g BSA, and 12.5 mL 20% Triton X-100. Two 15-minute blocks of PBS-AT were performed. Primary antibodies were diluted as follows: rabbit anti-human SERCA3 ATPase IgG (1:250) (ProteinTech, Cat. #13619-1-AP) or mouse anti-SERCA3 ATPase (1:100) (Abcam, Cat. #ab54876), guinea pig anti-human Insulin IgG (1:250) (Abcam, Cat. #GR185515-2), and rabbit anti-glucagon IgG (1:100) (Abcam, Cat. #ab8055) in PBS-AT. Primary antibody incubation was performed at room temperature for 1 hour. After this hour, the coverslips were washed ten times with the PBS-AT solution. The secondary antibodies were diluted as follows: anti-mouse Alexa 596 (1:1000) (Invitrogen, Cat. #A11029) or anti-rabbit Alexa 488 (1:1000) (Invitrogen, Cat. #A11034), and anti-guinea pig Alexa 610 (1:500) (Invitrogen, Cat. #A21450) in PBS-AT. The secondary antibody incubation was run for 1 hour at room temperature. After the incubation, 5 washes of PBS-AT and 5 washes of PBS were performed. The coverslips were mounted in Prolong Gold with Dapi (Life Technologies, Cat. #P36935). One drop was placed onto each slide before the coverslip was mounted cell side down. The slides with coverslips were left in the dark to dry overnight at room temperature, and then rimmed with nail polish. These coverslips were imaged for immunofluorescence using the Nikon Eclipse Ti Microscope and Nikon Elements Analysis software.

qRT-PCR

qPCR was conducted to assess the expression of mRNA within each of the samples. The RNA within each sample was isolated from harvested cells, pelleted, and lysed in 700 μ l QIAzol Lysis Reagent (Qiagen). RNA from human islets and cell lines was prepared using a Qiagen RNEasy[®] Mini Kit (Cat. #74104 & 74106). The isolation was performed according to manufacturer instructions. The samples were stored at -80°C. The concentration and quality of RNA was determined using the Bioanalyzer at UMass Medical School Core Facility.

To prepare cDNA, SuperScript ViLo Master Mix was used to generate cDNA from the isolated RNA (Invitrogen, Cat. #11755-050). The isolated cDNA was used to perform qPCR for the gene of interest using a Taqman Probe Assay. For SERCA3, a probe was used for the gene ATP2A3 (Applied Biosystems, Cat. #hs00193090). Actin was used as a control (Applied Biosystems, Cat. #hs01060665).

Viral Knockdowns and Cell Culturing

Adenoviral mediated knockdowns using RNAi technology were performed using EndoC β H-1 human pancreatic cells, as well as stem cell derived human β -cells from Melton Lab (Pagliuca et al., 2014). EndoC β H-1 Human Pancreatic Cells were grown in cultures for a minimum of 2 weeks to reach the desired confluence and cell count. Media were changed once a week to encourage cell growth by removing 10 mL of the growth medium and replacing it with 10 mL of pre-warmed, fresh growth medium. The stem cell derived β -cells were generously provided by Melton Lab (Harvard University).

For Adenoviral knockdowns, the cells were plated on 6-well well plates or coverslips, and infected with 50, 100, or 200 MOI of appropriate adenovirus. Adenovirus

infection was performed for 48 and 72 hours to determine maximum knockdown efficiency for SERCA3. The adenovirus SERCA3 construct and GFP control were purchased from Vigene. To determine infectivity of the virus, each construct expressed GFP to mark infected cells. Following infection, the cells were harvested for Western analysis and qPCR to determine knockdown efficiency, and coverslips were fixed for imaging. In addition, the stem cell derived beta cells were used for perfusion to determine the effect of knockdown on insulin secretion.

Immunofluorescent and Immunohistochemistry Staining

nPOD pancreatic tissues were stained by the Cell Morphology Core Facility at UMass Medical School. The Nikon Eclipse Ti Microscope was used to view the stained cells at a total magnification of 20x; which were selected for further observation and analysis. To accurately separate the view of the different proteins of interest in each image, four different filters were chosen: Dapi (Blue), FitC (Green), MCherry (Red), and Cy5 (Far Red). The fluorescence mean intensities were obtained and analyzed using Nikon Elements Analysis software. Exposure times were kept constant between samples in order to compare the intensities. Dapi was used as control for staining quality of samples and to normalize the mean intensities of light from the FitC and Cy5 filters. Approximately 10 islets were chosen from each pancreatic tissue sample.

Quantitative analysis was done in Microsoft Excel to compare overall fluorescence intensity values for each filter. The FitC (SERCA3) and Cy5 (Insulin) were normalized to Dapi and a ratio was obtained for the average intensity of Fitc/Dapi and Cy5/Dapi. This data was used to extrapolate differences in levels of Insulin and Serca3 in healthy and T2D patients.

Western Blotting

Cells were harvested using a cell scraper in 100-200 μ L of RIPA Lysis buffer (Boston BioProducts, Cat. #bp-115) with Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Cat. #11374600) and lysed in ice for 20 minutes with frequent pipetting. Protein concentration was determined using a Pierce® BCA Protein Assay Kit (ThermoScientific Cat. #23225) according to the manufacturer's protocol. 10 μ g of protein was loaded onto each well of 4-20% gradient gel and run at 100 volts for 15 minutes, and then up to 170 volts until completion. The gel was transferred for 2 hours at 100 volts onto a PVDF membrane. For transfer, the gel was equilibrated in transfer buffer. A "transfer sandwich" was prepared that contained filter paper, a PVDF membrane, and gel, and was cushioned by pads and pressed together by a support grid. Before assembly, the membrane was saturated with 100% methanol to wet the membrane and allow for appropriate transfer of the proteins. After assembling the sandwich, it was placed vertically in an electrophoresis tank between wire electrodes and filled with transfer buffer. To ensure that the gel would not overheat due to the high voltage passing through the apparatus, it was surrounded in an ice bath.

After transfer, the membrane was blocked in 5% milk, followed by incubation with primary polyclonal antibodies: rabbit or mouse anti-human SERCA3 (1:500) (ProteinTech, Cat. #13619-1-AP), rabbit anti-human insulin (1:250) (Abcam, Cat. #ab7842), and mouse anti-human Actin (1:3000) (Abcam, Cat. #ab8055). The primary antibody incubated for at least 12 hours, but no more than 24 hours, at 4°C. The secondary antibodies were: goat anti-rabbit-HRP IgG (1:3000) (Invitrogen, Cat. #A11034), and rabbit anti-mouse HRP IgG (1:5000) (Invitrogen, Cat. #A11061). Secondary antibody incubations were run 1-2 hours at room temperature. Milk was used to block with non-specific proteins. Membranes were

developed using ECL Western Blotting Detection Reagent (Amersham, Cat. #9622301).

The film was developed with varying exposure times that depended on the protein signals.

RESULTS

The worldwide prominence of T2D has brought it the foreground of attention in the scientific community. T2D patients usually have insulin resistance, a lack of response to insulin, or lack insulin altogether, and tend to develop hyperglycemia. Although much research has focused on the mechanisms of insulin secretion, factors leading to insulin insensitivity are not fully understood. Insulin has been identified as the only hormone that lowers serum glucose levels in the body. When insulin binds to its target receptor, it initiates multiple signaling pathways that function in general gene expression, glucose metabolism, and protein synthesis. Disruption in these cell signaling pathways can result in glucose dysregulation, and thus, diabetes. One specific pathway that has previously been identified as important in Ca^{2+} signaling is the SERCA3 pathway. This pathway appears to be down regulated in T2D, but it is not clear whether its down-regulation is a cause or consequence of the disease. Thus, the experiments in this project were performed to examine the role of SERCA3 in insulin secretion, and its plausible correlation with down-regulation of insulin in T2D patients.

We began by using a proteome analysis to validate previous literature that SERCA3 protein is reduced in T2D patient Islets (N=9) compared to healthy controls (N=6) (**Figure-4**). The data show that SERCA3 protein is significantly reduced ($p=0.0071$) in T2D patient islets relative to healthy controls, and it supports and validates the previous findings.

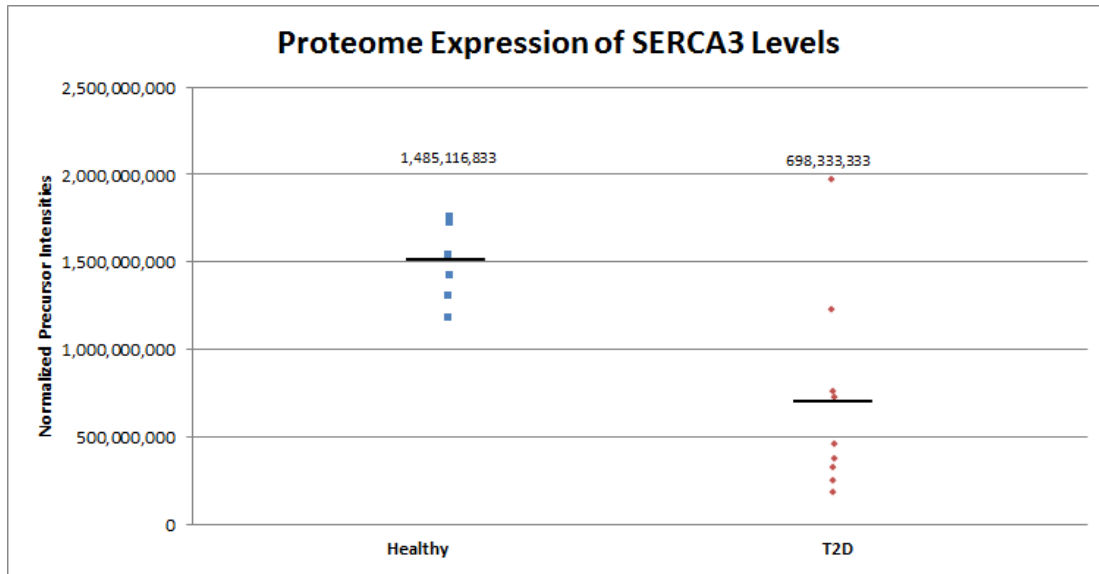


Figure-4: Quantification of Normalized Intensities for Protein Expression of SERCA3 Levels in Healthy Individuals (N=6) Compared to T2D Individuals (N=9). Average intensities were calculated for Healthy: 1,485,116,833, and T2D: 698,333,333. Statistical analysis gave a significant p-value of 0.0071.

Human β -Cell Immunohistochemical Staining Shows Decreased of SERCA3 in T2D Patients

Immunohistochemical staining and microscopy was performed with insulin or SERCA3 antibodies in healthy or diabetic pancreas sections to determine protein levels of these two key proteins. Pancreatic sections from normal or diabetic patients were stained and imaged using the Nikon Eclipse Ti Microscope (**Figure 5**). T2D patients had smaller islets, with significant down-regulation of insulin and SERCA3. Cell staining was performed using specimens from 5 (N=5) healthy patients, and 8 specimens (N=8) from T2D patients. These qualitative beta cell staining results indicate that the levels of insulin and SERCA3 proteins are decreased in T2D patients.

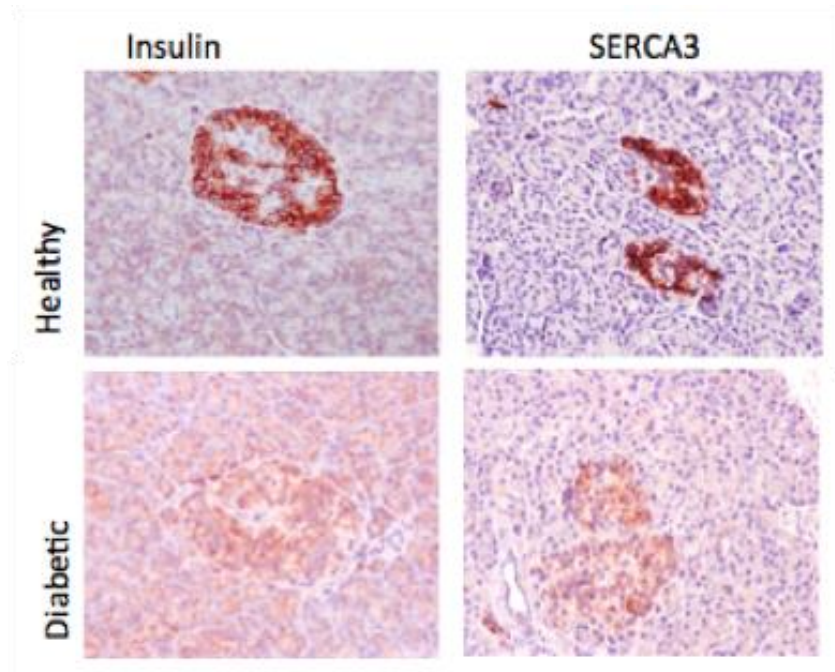


Figure 5: Example Insulin and SERCA3 Immunohistochemical Microscopy on Healthy and T2D Patients. Human pancreatic islet cells from healthy (upper row) or T2D patients (lower row) were stained using antibodies for insulin (left column) or SERCA3 (right column). T2D islets show significant down-regulation of insulin and SERCA3 proteins. This experiment was repeated several times, healthy N=5 and T2D N=8.

Insulin and SERCA3 Immunofluorescence

The down-regulation of SERCA3 observed with immunohistochemistry was further verified by immunofluorescence imaging (**Figure-6**). The figure shows a typical islet image of Cy5 (red) (insulin), FitC (green) (SERCA3), and the overlay of both (yellow). The healthy islet (upper row) is not morphologically compromised, and shows significant overlay of insulin and SERCA3. The T2D islet (lower row) is more dispersed, less concentrated, and the protein signals are reduced especially for SERCA3.

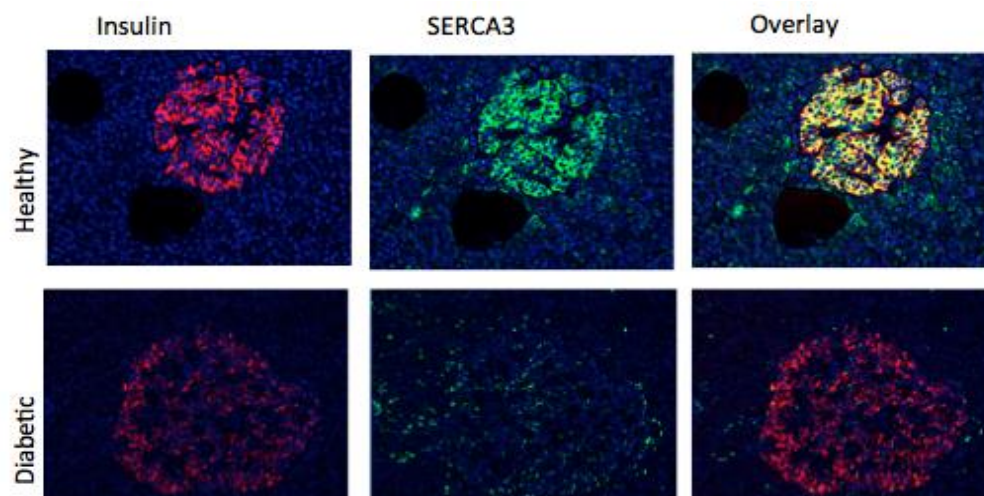


Figure-6: Example Immunofluorescent Micrograph of SERCA3 and Insulin Protein Levels in Healthy and T2D Human Pancreatic Islets. Dapi (blue) represents nuclei, Cy5 (red) denotes Insulin, and FitC (green) denotes SERCA3. T2D islets (bottom row) show lower levels of insulin and SERCA3 compared to control islets (top row). The overlay of insulin and SERCA3 appears normal in healthy islets, but with little to no overlap in T2D islets.

A second immunofluorescent micrograph is shown in **Figure 7** for one healthy sample and two T2D samples. Again, strong correlations were observed between the FitC (SERCA3) and Cy5 (Insulin) staining in the control islet, while the equivalent staining in T2D islets is reduced, and not localized in the same regions.

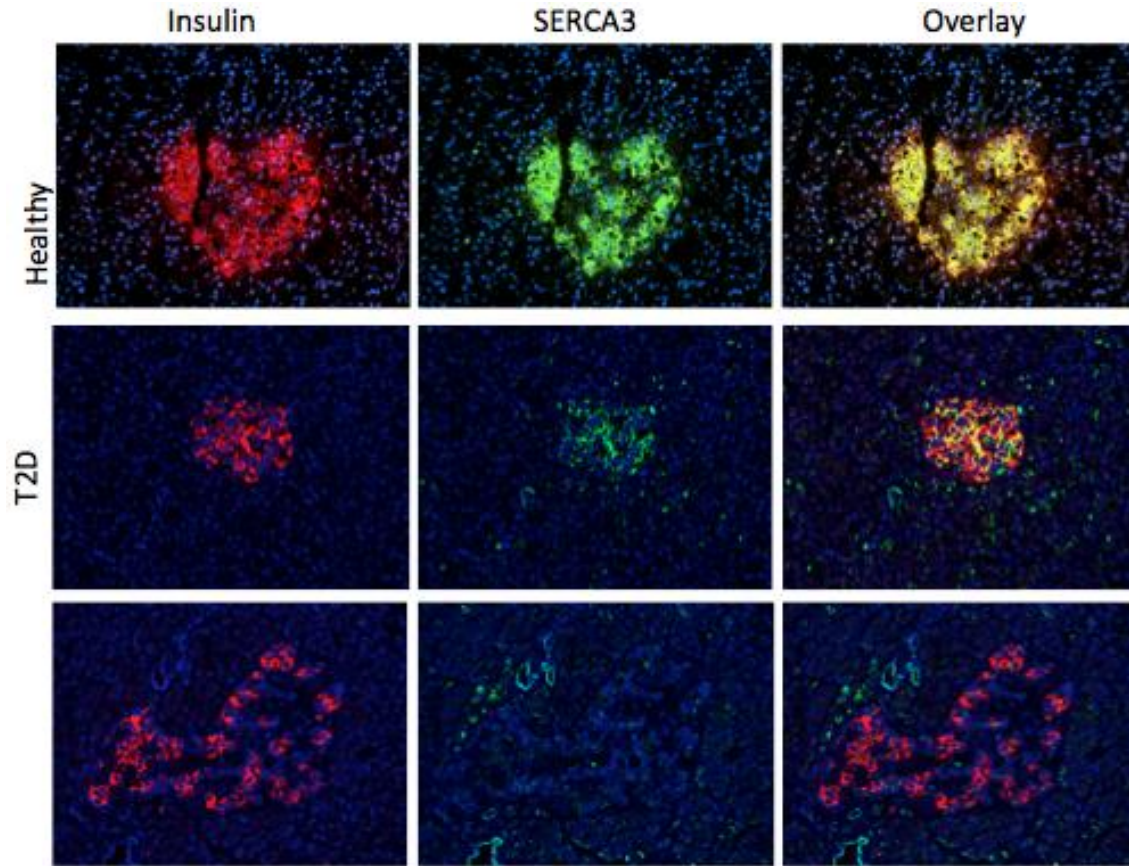


Figure-7: A Repeat Immunofluorescent Micrograph of Healthy and T2D Human Pancreatic Islets Stained for Insulin and SERCA3. Dapi (blue) stained nuclei, Cy5 (red) stained Insulin, and FitC (green) stained for SERCA3. T2D islets (bottom row) show lower levels of insulin and SERCA3 compared to control islets (top row). The overlay of insulin and SERCA3 appears normal in healthy islets, while little to no overlap (yellow) is present in diabetic islets.

The SERCA3 and insulin protein levels were quantitated from the immunofluorescent micrographs. Healthy individuals (N=101) had an average mean intensity of 5544 intensity units for SERCA3, and 3317 intensity units for Insulin. T2D individuals (N=84) had an average mean intensity of 4099 intensity units for SERCA3 and 2799 intensity units for Insulin. When normalized to Dapi, in order to adjust for differences in staining penetration and sample quality, the Healthy SERCA3/DAPI ratio was 1.812, compared to a T2D ratio of 1.218 (**Figure-8**). The Healthy Insulin/DAPI ratio was 1.092, compared to a T2D ratio of 0.813 (**Figure-9**). This data indicates that both SERCA3 and

insulin are lower in T2D patients compared to Healthy, and support the findings of the immunohistochemistry and western blots.

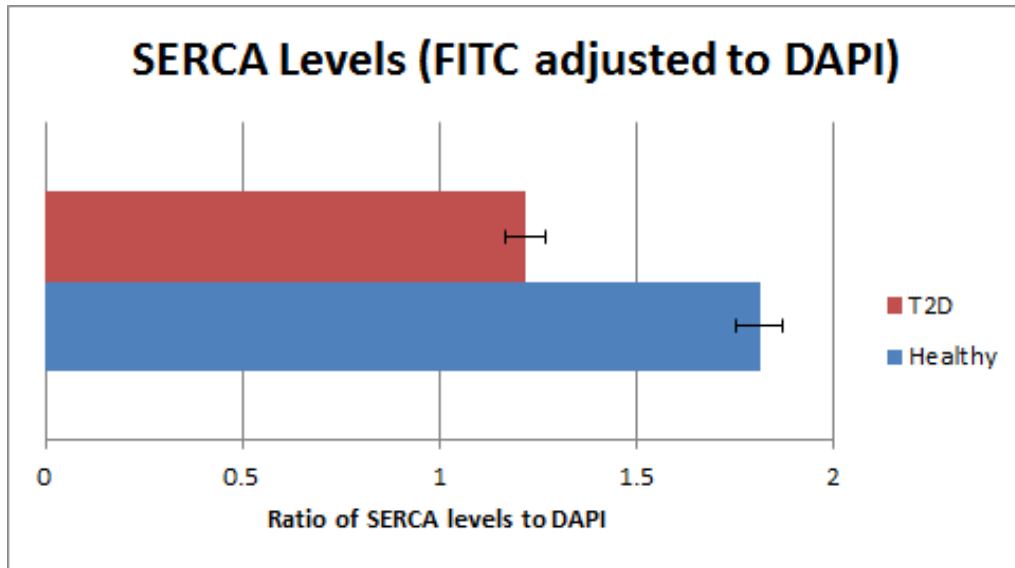


Figure-8: Quantitative Summary of the SERCA3 Fluorescence Levels of Figure-7. Shown are the mean intensity of SERCA3 levels (FitC) normalized to nuclei (Dapi) in T2D samples (red) and healthy samples (blue). The Healthy SERCA3/DAPI ratio is 1.812 ± 0.059 , while the same ratio in T2D patients is 1.218 ± 0.053 .

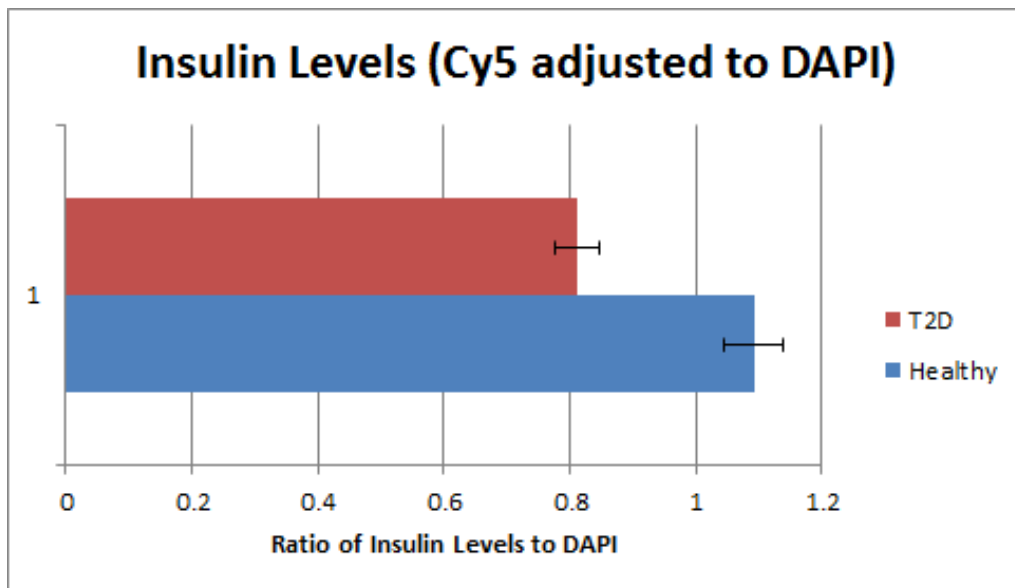


Figure-9: Quantitative Summary of the Insulin Data from Figure-7. Shown are the mean intensities of Insulin levels (Cy5) normalized relative to nuclei (Dapi), in diabetic samples (red) and healthy samples (blue). The mean Insulin/DAPI ratio in Healthy individuals is 1.092 ± 0.047 , while the same ratio in T2D patients is 0.813 ± 0.035 .

Taken together, the immunohistochemical and immunofluorescence data indicate that SERCA3 and Insulin protein levels are decreased in T2D samples compared to healthy individuals. The total intensities of 101 Healthy and 84 T2D pancreatic islets were quantified from 5 Healthy and 8 T2D patients. Statistical two-tailed t-tests were then conducted. The p value for SERCA3/DAPI, Healthy versus T2D, was 2.01×10^{-8} , indicating that SERCA3 is significantly lower in T2D compared to Healthy patients. The p value for Insulin/DAPI, Healthy versus T2D, was 6.26×10^{-3} , also showing a significantly lower protein level in T2D patients.

SERCA3 Western Blots

To support the qualitative and quantitative findings of the immunofluorescence microscopy, SERCA3 western blots were performed to determine the protein levels (**Figure 10**). The western blot shows N=3 healthy samples compared to N=4 T2D samples, and again shows a reduction in T2D SERCA3 protein levels relative to healthy control protein levels. Huge differences of variability in levels of Actin and Insulin within just the T2D samples has led to problematic statistical analysis. Also, because the imaged bands of SERCA3, Insulin, and Actin were stitched together, each had a different background lighting, thus affecting the average band intensity of each sample. Because of this, the insulin signals were not quantified from this blot, but given the higher protein load for the two T2D lanes this indicates that insulin may also be reduced in the T2D samples.

The data is quantified in (**Table-4**), and shows no significant correlation between SERCA3 and insulin bands from healthy and T2D patients. It is suspected that the huge variability in band intensity resulted in high deviations and a larger p-value. The actin bands indicate that the loading of each well was not equal, which is further proved by the

difference in Insulin levels between T2D samples 1 and 2, vs 3 and 4. With a p-value of 0.1018, the data from Table 4 and the western blot in Figure 11 is statistically insignificant.

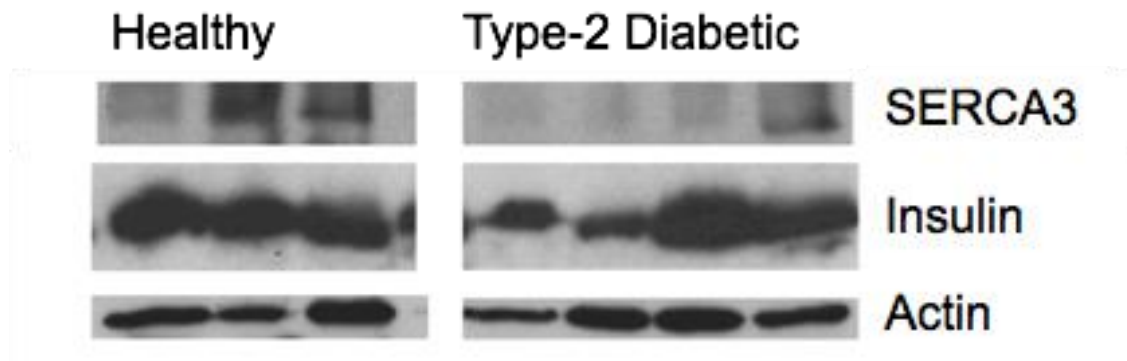


Figure-10: Repeat SERCA3 and Insulin Western Blot Showing N=3 WT Samples and N=4 T2D Samples. Bands are shown for SERCA3 (top row), Insulin (middle row), and actin (lower row). Approximate band lengths are: SERCA3, 97 kD; Actin, 42 kD; Insulin, 6 kDa.

Table 4: Mean SERCA3 Band Intensities from Figure-6 Blot.

	Healthy Individuals	T2D Patients
Sample-1	123.73	98.28
Sample-2	176.92	97.91
Sample-3	163.16	105.48
Sample-4		146.61
Average SERCA3 Band Intensity Relative to Actin	1.069	0.779
STDev (%)	20.6%	19.8%
Std. Err (%)	12.7%	7.7%
P Value	0.1018	

Adenoviral Knockdown Experiments for SERCA3

In order to perform a knockdown of SERCA3 in pancreatic cells, EndoC- β H1 cells and human stem cell derived β -cells were infected with an adenovirus encoding a SERCA3 shRNA. The adenovirus also encoded green fluorescent protein (GFP) as a marker for infected cells. Adenoviral infection was tested for several variables, including: length of incubation and titer of adenovirus. The data indicated that SERCA3 islets were equally susceptible to infection by Adeno-GFP and Adeno-shRNA-GFP.

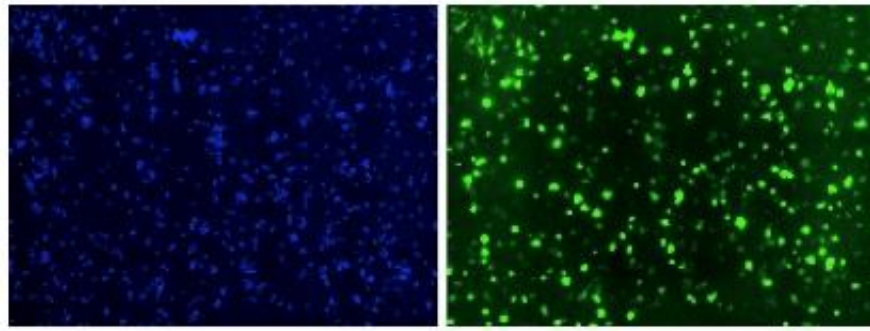


Figure-11: Percent Infection of Pancreatic EndoC- β H1 Cells With Adenovirus. The Adeno-GFP signal shows the presence of adenoviral-infected cells. After 72 hours of infection, the cells show about 90% infection efficiency.

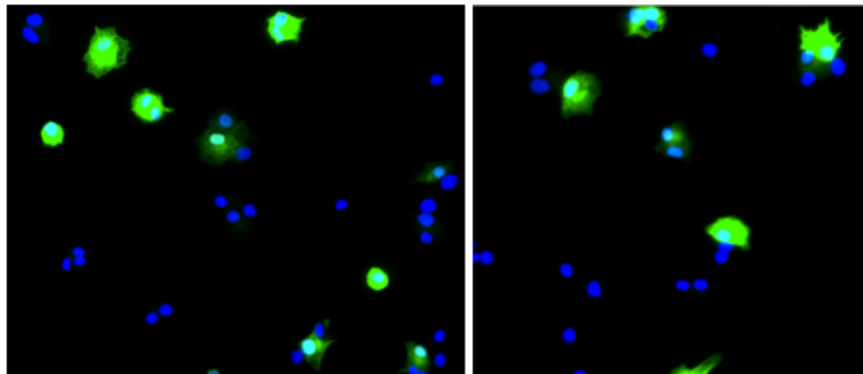


Figure-12: Comparison of Infection Rates of EndoC- β H1 Cells with Adeno-GFP Control (Adenovirus encoding GFP but no snRNA) versus Cells Infected With Adenovirus-shRNA-GFP (MOI of 200). The green Adeno-GFP signal (left) shows the presence of GFP tagged cells. The Adeno-shRNA-GFP green signal (right) also shows the presence of adenoviral-infected cells. After 72 hours of infection, both the control and shRNA-infected cells show similar rates of infection.

The successfully infected EndoC- β H1 cells can then be used in future assays to perform further phenotypic analysis for SERCA3 and Insulin.

qRT-PCR was conducted to assess the amount of SERCA3 mRNA present in control and experimental samples. Calculated mRNA levels for SERCA3 were determined in the EndoC- β H1 cells for both Adeno-GFP control (adenovirus encoding GFP but no shRNA) and the shSERCA3 (knockdown) samples. Differences in fold change between the two sample types can be seen below (**Figure-14**). The fold change was normalized to actin control and was assigned number 1.0 for the Adeno-GFP cells. A fold change of 0.0625 was calculated for the shSERCA samples, an indicator of a successful knockdown of SERCA3 mRNA. Since there was only one sample, triplicate runs for qPCR could not provide statistical results, so this experiment needs to be repeated with additional samples in order to reach statistical significance.

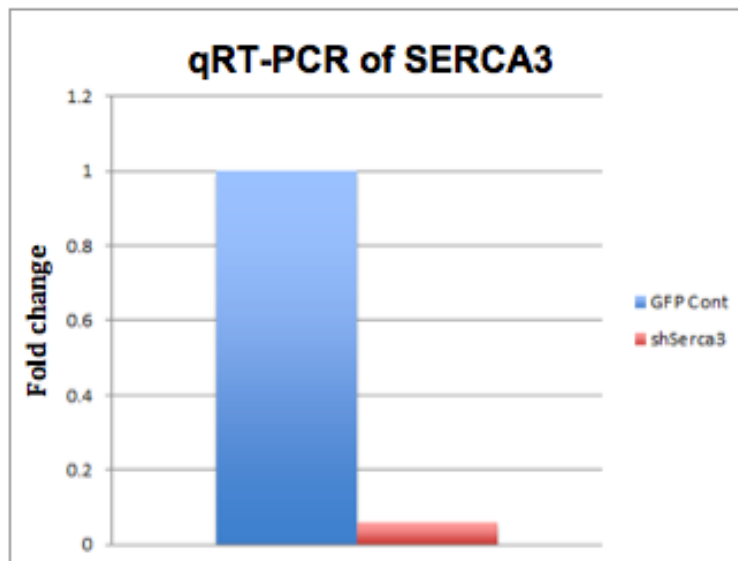


Figure-13: qRT-PCR Analysis of SERCA3 mRNA Levels for Adeno-GFP Control and Adeno-shSERCA3 Cells. qRT-PCR was conducted to quantify SERCA3 mRNA levels in our control and experimental samples (N=2). Fold changes were calculated for Adeno-GFP Control (1.00) and Adeno-shSERCA3 (0.0625).

SERCA3 Protein Knockdown

A western blot was run to determine the SERCA3 protein levels in control and knockdown cells (**Figure-14**). The figure shows a comparison of two control EndoC- β H1 cell line lysates versus two shSERCA lysates immunoblotted for SERCA3 (top band), Actin (middle band), and Insulin (bottom band), whose identities were deduced using specific antibodies and from their known relative kD protein sizes: SERCA3, 97 kD; Actin, 42 kD; Insulin monomer, ~6 kDa (Sen et al., 2002). It was quantified that slightly more actin was loaded into lanes 4 and 5, but those two samples still showed reduced SERCA3 and insulin protein levels relative to control lanes 2 and 3.

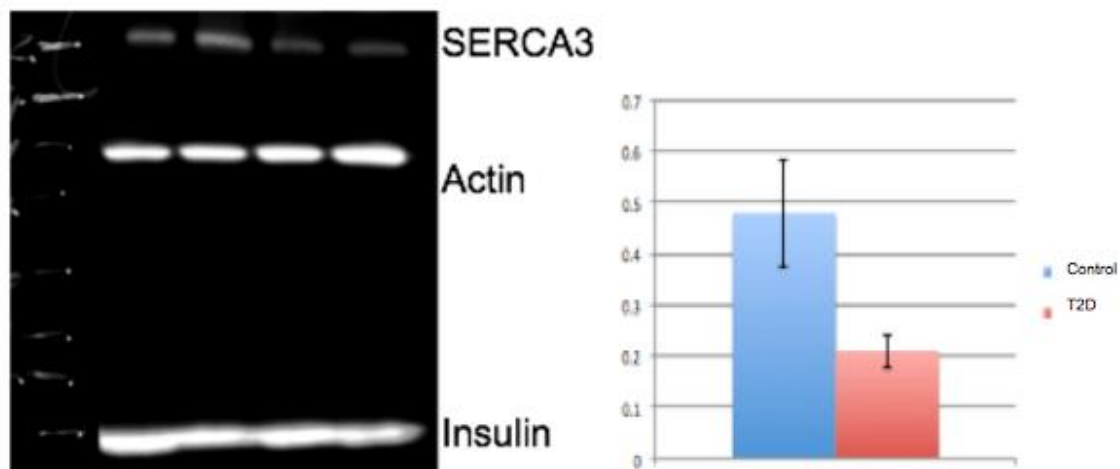


Figure 14: SERCA3 and Insulin Western Blot and Quantitation. The left panel shows a SERCA3 western blot, and the right panel shows quantification of the SERCA3 bands relative to actin. Blot lanes are: MW marker (lane 1), healthy controls (Lanes 2 and 3), and SERCA3 knockdown experimental samples (Lanes 4 and 5). Bands are SERCA3 (top), Actin (middle), and Insulin (bottom), identified by their expected MWs. The right panel shows a densitometer quantitation of the SERCA3 bands (N=2). Statistics was not applied to the plot, as it represents only two replicates.

The mean intensities of the SERCA3 bands were quantitated by densitometry relative to actin, and are shown in the right panel of (**Figure-14**) and in (**Table-5**). SERCA3 protein levels were approximately 56.2% lower in SERCA3 knockdown

lanes compared to Adeno-GFP control. Standard deviations were 31% and 22%, respectively, for control and shSERCA3. Standard error was 10.5% and 3.3%, respectively, for control and shSERCA3. The western data indicates that SERCA3 expression was reduced by the specific shRNAs.

**Table 5: Mean SERCA3 Band Intensity
From Figure-14 Blot.**

	Control	Knockdown
Sample-1	40.94	29.67
Sample-2	64.25	27.02
Average SERCA3 Band Intensity Relative to Actin	0.479599	0.210089204
STDev (%)	14.8976	4.6933901
Std. Err (%)	10.5342	3.318728
P Value	0.0123	

Statistical tests show significant differences in SERCA3 levels relative to actin in control samples versus knockdown samples. This data showing lower SERCA3 protein levels in Adeno-shRNA cells relative to Adeno-GFP cells supports the qRT-PCR data showing a similar result for SERCA3 mRNA.

DISCUSSION

The findings of this project provide statistically significant evidence that SERCA3 is down-regulated in T2D islets compared to healthy islets. This observation was consistent throughout immunohistochemical staining of pancreatic cells, western blots, and immunofluorescent microscopy. For the latter assay, the reduction was validated as being statistically significant in a total of 185 samples (N=101 WT; and N=84 T2D). The reduced levels of SERCA3 in T2D patient islets supports previous research arguing that disruption of Ca^{2+} signaling in pancreatic cells can lead to reduced insulin secretion and diabetic symptoms (Borge et al., 2002; Liang et al., 2014; Gilon et al., 2014). Previous work with diabetic mice showed an impaired SERCA3 function correlated with β -cell apoptosis (Varadi et al., 1999), and that down-regulation of SERCA3 can affect calcium homeostasis in pancreatic β -cells, which impairs normal insulin secretion (Zarain-Herzberg et al., 2014). In addition, Varadi et al. (1999) identified four rare missense mutations in exons 4, 14, and 15 in the SERCA3 gene of T2D patients with marked hyperglycemia and β -cell dysfunction.

Several problems were encountered during this project. One problem was the significant amount of time required for each experiment, from the initial culture of the cells to the final cell staining and signal analysis. Planning several weeks in advance was required to ensure that all experiments could be achieved. In addition, some of the protocol details had to be worked out, such as the choice of primary antibodies used in the experiments, and the proper incubation times. Specifically, while performing Western analysis a rabbit anti-human SERCA3 ATPase IgG (1:100) primary antibody was used initially. Since the rabbit antibody is polyclonal, its binding affinity may not be as specific

as the monoclonal mouse anti-SERCA3 ATPase (1:100) antibody and both were run to verify results. However, the rabbit anti-human SERCA3 ATPase IgG (1:100) antibody worked better in immunofluorescent imaging, and so this antibody was used instead of the mouse anti-SERCA3 ATPase (1:100) antibody in experimental repeats. Quantification of data from immunofluorescent imaging relied on accurate selection of regions of interest in Nikon Elements Analysis software. Human error and variation also contributed to standard error of quantitative results during this process. Another complication of IF imaging was that the pancreatic tissues were not physically flat, so some parts of the islets remained out of focus when selecting for ROIs. These unfocused regions could affect the calculations of Insulin and SERCA3 mean intensities.

Other complications aside from IF imaging include the membrane high background that was present in one of the Western Blots that was performed (Figure-11). Again, this may have caused discrepancies in the quantitative analysis of band intensities due to inconsistent background intensity or higher background intensity. The inconsistent loading as observed by unequal Actin (control) in the lanes may have skewed the quantification of SERCA3 and Insulin levels as well since the intensities were adjusted to the level of Actin. For this reason, this project included experimental repeats to minimize discrepancies due to human error and standard deviation.

Several future experiments would nicely extend this preliminary project. Given the successful knockdown of SERCA3 by shRNA, shown by results of Western analysis and qRT-PCR, the next step in determining whether the SERCA3 pathway is required for optimal insulin secretion would be to assay insulin levels in the knockdown cells relative to controls using westerns and RT-PCR. Due to time restraints, the knockdowns were not optimized to minimize expression, and they were not analyzed for insulin secretion assays.

After using an adenovirus to perform knockdowns, a lentiviral knockdown system could then be used as a second method of verification. A lentiviral knockdown would provide additional information that an adenoviral knockdown could not. Adenoviral DNA does not integrate into DNA and therefore is not copied during cell replication; in contrast, lentiviral DNA is passed on during cell replication. Both knockdown systems could be compared for efficiency of knockdown. In addition, CRISPR technology, a novel technique used for efficient gene knockouts, could also be tested to alter the genome to eliminate the SERCA3 gene. The gene removal could be verified by genomic PCR, and its effects on SERCA3 expression measured by qRT-PCR and westerns.

The next steps after a confirmed knockdown would be to test the effects of the knockdown on insulin secretion *in vitro* (by perfusion) and *in vivo* (by transplantation). These techniques would help determine whether any change in hormonal levels are a cause or consequence of SERCA3 knockdown. For instance, if insulin secretion was lowered by a SERCA3 knockdown, then this provides evidence that the SERCA3 metabolic pathway is required for optimal insulin secretion and could relate to the pathogenesis of T2D. However, if insulin secretion was not lowered in knockdown cells, then this provides evidence that optimal insulin secretion does not require proper functioning of the SERCA3 metabolic pathway and that downregulation of SERCA3 must be a consequence of the pathogenesis of T2D.

BIBLIOGRAPHY

Arredouani A, Guiot Y, Jonas JC, Liu LH, Nenquin M, Pertusa JA, Rahier J, Rolland JF, Shull GE, Stevens M, Wuytack F, Henquin JC, Gilon P (2002) SERCA3 ablation does not impair insulin secretion but suggests distinct roles of different sarcoendoplasmic reticulum Ca(2+) pumps for Ca(2+) homeostasis in pancreatic beta-cells. *Diabetes*, 51(11): 3245-3253.

Bai M, Fan M, Oh E, & Wang Z (2012) A Triple Helix-Loop-Helix/Basic Helix-Loop-Helix Cascade Controls Cell Elongation Downstream of Multiple Hormonal and Environmental Signaling Pathways in Arabidopsis. *The Plant Cell*, 24(12): 4917-4929. doi:10.1105/tpc.112.105163

Borge PD, Moibi J, Greene SR, Trucco M, Young RA, Gao Z, & Wolf BA (2002) Insulin Receptor Signaling and Sarco/Endoplasmic Reticulum Calcium ATPase in B-Cells. *Diabetes*, 51 (Supplement 3): S427-S433.

Chen J, Chang L, Wu C, Yeung L, & Lin Y (2011) Involvement of F-Actin in Chaperonin-Containing t-Complex 1 Beta Regulating Mouse Mesangial Cell Functions in a Glucose-Induction Cell Model. *Experimental Diabetes Research*, 2011: 1-10.

Cong Y, Baker ML, Jakana J, Woolford D, Miller EJ, Reissmann S, Kumar RN, Redding-Johanson AM, Batth TS, Mukhopadhyay A, Ludtke SJ, Frydman J, Chiu W (2010) 4.0-A resolution cryo-EM structure of the mammalian chaperonin TRiC/CCT reveals its unique subunit arrangement. *Proceedings of the National Academy of Sciences, USA*, 107(11): 4967-4972.

Diiorio P, Jurczyk A, Yang C, Racki WJ, Brehm MA, Atkinson MA, Powers AC, Shultz LD, Grenier DL, Bortell R (2011) Hyperglycemia-Induced Proliferation of Adult Human Beta Cells Engrafted Into Spontaneously Diabetic Immunodeficient NOD-Rag1null IL2rynull Ins2Akita Mice. *Pancreas*, 40(7): 1147-1149.

Diiorio P, Rittenhouse AR, Bortell R, & Jurczyk A (2014) Role of cilia in normal pancreas function and in diseased states. *Birth Defects Research Part C: Embryo Today: Reviews Birth Defect Res C*, 102(2): 126-138.

Dode L, Wuytack F, Kools PF, Baba-Aissa F, Raeymaekers L, Brike F, van de Ven WJ, Casteels R (1996) cDNA cloning, expression and chromosomal localization of the human sarco/endoplasmic reticulum Ca²⁺-ATPase 3 gene. *Biochemical Journal*, 318 (2): 689-699.

Fonatsch C, Gradl G, Ragoussis J, Ziegler A (1987) Assignment of the TCP1 locus to the long arm of human chromosome 6 by in situ hybridization. *Cytogenetics and Cell Genetics*, 45 (2): 109-112.

Gilon P, Chae H, Rutter GA, & Ravier MA (2014) Calcium signaling in pancreatic β -cells in health and in Type 2 diabetes. *Cell Calcium*, 56(5): 340-361.

Harris L (2013) Bardet-Biedl Syndrome 2 (bbs2) Depletion Affects Primary Cilia. In: Pancreatic Islets and Glucose Homeostasis.

Jensen J, Heller RS, Funder-Nielsen T, Pedersen EE, Lindsell C, Weinmaster G, Madsen, OD, Serup P (2000) Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: A role for the notch pathway in repression of premature differentiation. *Diabetes*, 49(2), 163-176. doi:10.2337/diabetes.49.2.163

Jurczyk A, Diiorio P, Brostowin D, Leehy L, Yang C, Urano F, Harlan DM, Shultz LD, Greiner DL, Bortell R (2013) Improved function and proliferation of adult human beta cells engrafted in diabetic immunodeficient NOD-scid IL2 γ null mice treated with alogliptin. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy DMSO*, 6: 493-499.

Jurczyk A, Bortell R, & Alonso LC (2014) Human β -cell regeneration: progress, hurdles, and controversy. *Current Opinion in Endocrinology & Diabetes and Obesity*, 21(2): 102-108.

Jurczyk A, Nowosielska A, Przewozniak N, Aryee KE, DiIorio P, Blodgett D, Yang C, Campbell-Thompson M, Atkinson M, Shultz L, Rittenhouse A, Harlan D, Greiner D, Bortell R (2015) Beyond the brain: Disrupted in schizophrenia 1 regulates pancreatic β -cell function via glycogen synthase kinase-3. *FASEB Journal*, 30(2): 983-993.

Liang K, Du W, Lu J, Li F, Yang L, Xue Y, Hille B, and Chen L (2014) Alterations of the Ca^{++} signaling pathway in pancreatic beta-cells isolated from db/db mice. *Protein Cell*, 5(10): 783-794.

Nakatani Y, Kaneto H, Kawamori D, Yoshiuchi K, Hatazaki M, Matsuoka TA, Ozawa K, Ogawa S, Hori M, Yamasaki Y, Matsuhisa M (2005) Involvement of endoplasmic reticulum stress in insulin resistance and diabetes. *Journal of Biological Chemistry*, 280(1): 847-851.

Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Görgün C, Glimcher LH, Hotamisligil GS (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science*, 306(5695): 457-461.

Ozawa K, Miyazaki M, Matsuhisa M, Takano K, Nakatani Y, Hatazaki M, Tamatani T, Yamagata K, Miyagawa J, Kitao Y, Hori O, Yamasaki Y, Ogawa S (2005) The endoplasmic reticulum chaperone improves insulin resistance in type 2 diabetes. *Diabetes*, 54(3): 657-663.

Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Görgün CZ, Hotamisligil GS (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science*, 313(5790): 1137-1140.

Pagliuca F, Millman J, Gürtler M, Segel M, Van Dervort A, Ryu J, Melton D (2014) Generation of Functional Human Pancreatic β Cells In Vitro. *Cell*, 159(2): 428-439.

Ravier MA, Daro D, Roma LP, Jonas JC, Cheng-Xue R, Schuit FC, Gilon P (2011) Mechanisms of Control of the Free Ca^{++} Concentration in the Endoplasmic Reticulum of Mouse Pancreatic Cells: Interplay With Cell Metabolism and $[\text{Ca}^{++}]_c$ and Role of SERCA2b and SERCA3. *Diabetes*, 60(10): 2533-2545.

Rorsman P, and Braun M (2013) Regulation of insulin secretion in human pancreatic islets. *Annual Review of Physiology*, 75: 155-179.

Saltiel AR and Kahn CR (2001) *Nature*, 414: 799-806.

Sen A, Daly ME, & Hui SW (2002) Transdermal insulin delivery using lipid enhanced electroporation. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1564(1): 5-8.

Seo S, Baye LM, Schulz NP, Beck JS, Zhang Q, Slusarski DC, Sheffield VC (2010) BBS6, BBS10, and BBS12 form a complex with CCT/TRiC family chaperonins and mediate BBSome assembly. *Proceedings of the National Academy of Science, USA*, Jan 26; 107(4): 1488-1493.

Slavotinek AM, & Biesecker LG (2001) Unfolding the role of chaperones and chaperonins in human disease. *Trends in Genetics*, 17(9): 528-535.

Spiess M, Echbarthi M, Svanström A, Karlsson R, & Grantham J (2015) Over-Expression Analysis of All Eight Subunits of the Molecular Chaperone CCT in Mammalian Cells Reveals a Novel Function for CCTdelta. *Journal of Molecular Biology*, 427(17): 2757-2764.

TCP1 t-complex [homo sapiens] genes and mapped phenotypes. (n.d.). Retrieved February 13, 2016, from <http://www.ncbi.nlm.nih.gov/gene/6950>.

Varadi A, Lebel L, Hashim Y, Mehta Z, Ashcroft SJ, Turner R (1999) Sequence variants of the sarco(endo)plasmic reticulum Ca^{++} -transport ATPase 3 gene (SERCA3) in Caucasian Type II diabetic patients (UK Prospective Diabetes Study 48). *Diabetologia*, 42(10): 1240-1243.

Wellen KE, and Hotamisligil GS (2005) Inflammation, stress, and diabetes. *Journal of Clinical Investigation*, 115(5): 1111-1119.

Williams R (2011) Williams Textbook of Endocrinology (12th ed., pp. 1371-1435). Philadelphia: Elsevier/Saunders.

Willison K, Kelly A, Dudley K, Goodfellow P, Spurr N, Groves V, Gorman P, Sheer D, Trowsdale J (1987) The human homologue of the mouse t-complex gene, TCP1, is located on chromosome 6 but is not near the HLA region. *EMBO Journal*, 6 (7): 1967-1974.

Xu GG, Gao Z, Borge PD, Jegier PA, Young RA, & Wolf BA (2000) Insulin Regulation of β -Cell Function Involves a Feedback Loop on SERCA Gene Expression, Ca^{2+} Homeostasis, and Insulin Expression and Secretion. *Biochemistry*, 39(48): 14912-14919.

Zarain-Herzberg A, García-Rivas G, Estrada-Avilés R (2014) Regulation of SERCA pumps expression in diabetes. *Cell Calcium*, 56(5): 302-310.